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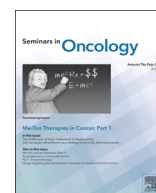
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Guidance Statement On *BRCA1/2* Tumor Testing in Ovarian Cancer Patients



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ABSTRACT

The approval, in 2015, of the first poly (adenosine diphosphate-ribose) polymerase inhibitor (PARPi; olaparib, Lynparza) for platinum-sensitive relapsed high-grade ovarian cancer with either germline or somatic *BRCA1/2* deleterious mutations is changing the way that *BRCA1/2* testing services are offered to patients with ovarian cancer. Ovarian cancer patients are now being referred for *BRCA1/2* genetic testing for treatment decisions, in addition to familial risk estimation, and irrespective of a family history of breast or ovarian cancer. Furthermore, testing of tumor samples to identify the estimated 3%–9% of patients with somatic *BRCA1/2* mutations who, in addition to germline carriers, could benefit from PARPi therapy is also now being considered. This new testing paradigm poses some challenges, in particular the technical and analytical difficulties of analyzing chemically challenged DNA derived from formalin-fixed, paraffin-embedded specimens. The current manuscript reviews some of these challenges and technical recommendations to consider when undertaking *BRCA1/2* testing in tumor tissue samples to detect both germline and somatic *BRCA1/2* mutations. Also provided are considerations for incorporating genetic analysis of ovarian tumor samples into the patient pathway and ethical requirements.

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1. Introduction

1.1. *BRCA1/2*: Associated cancer risk and genetic testing

BRCA1 (OMIM#113705) and *BRCA2* (OMIM# 600185) genes (chromosome 17q21 and 13q12, respectively) [1,2] are tumor suppressor genes encoding proteins primarily involved in DNA repair. Loss of function mutations in *BRCA1/2* are associated with

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increased risk of breast cancer and ovarian cancer (BC, OC) and to a lesser extent other cancers (eg, prostate cancer) [3,4]. Women carrying a pathogenic germline mutation in either *BRCA1* or *BRCA2* have a substantially increased lifetime risk of developing BC and OC: 40%–85% lifetime BC risk with *BRCA1/2* mutations, with higher rates in *BRCA1* mutation carriers [5,6]. Similarly, for OC the lifetime risk is estimated to be higher in women inheriting a *BRCA1* mutation (25%–65%) compared with a *BRCA2* mutation (15%–20%) [5,6]. The precise cancer risk in female *BRCA1/2* germline mutation carriers and the factors that govern mutant allele penetrance depend on many factors, including personal and/or reproductive factors, mutation location, and as yet undefined genetic factors ('modifier genes') [7,8].

Pathogenic *BRCA1/2* variants are distributed along the entire gene coding regions and overwhelmingly result in truncation and/or inactivation of the mutant protein. Genetic variants include nonsense, frameshift, splice-site, and some missense mutations, as well as large deletion duplications and rearrangements [9,10]. Since the cloning of *BRCA1/2* genes, individuals with a strong family history of BC and/or OC have undergone oncogenetic counseling and *BRCA1/2* genotyping to identify germline mutation carriers. In families where a pathogenic mutation is detected, the mutation serves as a relevant tool for assessing lifetime cancer risk. Asymptomatic, cancer-free female mutation carriers are offered surveillance for early BC detection and the possibility of risk-reducing surgery [11]. Mutation-carrying women are recommended to undergo risk-reducing salpingo-oophorectomy between 35–40 years of age (after childbearing) because of the lack of a proven surveillance scheme impacting on survival for the early detection of OC.

In 2015, the first poly (adenosine diphosphate-ribose) polymerase inhibitor (PARPi; olaparib, Lynparza) was licensed for the treatment of *BRCA*-mutated OC. In a Phase II clinical trial involving 265 patients with either platinum-sensitive recurrent ovarian, fallopian tube or primary peritoneal (high-grade) cancer, maintenance therapy with olaparib showed an improvement in median progression-free survival (PFS). This improvement was more pronounced in patients whose tumors had *BRCA1/2* mutations; hazard ratio (HR) 0.18 compared with wild-type *BRCA1/2* patients HR 0.54 [12]. More recently, results of the phase III trial (SOLO-2; N = 295) of olaparib tablets as maintenance therapy in patients with relapsed germline-mutated *BRCA1/2* OC reported an HR of 0.25 for PFS of patients receiving olaparib versus placebo, and an HR of 0.50 for time to second progression or death for the olaparib versus placebo group [13]. In another Phase III trial of the PARPi niraparib (the European Network of Gynaecological Oncology Trial groups (ENGOT)-OV16/NOVA trial) involving 553 OC patients, an HR of 0.27 for PFS was observed for niraparib treatment compared with placebo in OC patients with germline *BRCA1/2* mutations; HR 0.45 for PFS in wild-type *BRCA1/2* mutation group and HR 0.38 for patients with germline wild-type *BRCA1/2* patients with homologous recombination deoxyribonucleic acid (DNA) repair deficient (HRD) tumors [14].

Poly (adenosine diphosphate-ribose) polymerase (PARP) enzymes function in the repair of single-stranded DNA (ssDNA) breaks via the base-excision repair pathway [15,16]. Therefore, PARP inhibition results in an accumulation of ssDNA breaks; in normal cells these become converted to double-stranded DNA (dsDNA) breaks, which are repaired by the homologous recombination repair (HRR) pathway [15]. In cells lacking a functioning *BRCA1/2* protein, repair by the HRR pathway is also inhibited; therefore, PARPi treatment of *BRCA1*- and *BRCA2*-associated cancers causes 'synthetic lethality' (when two non-lethal processes combine to cause cell death) specifically to cancer cells [15,17,18].

Olaparib is licensed in the European Union as maintenance therapy for adult patients with platinum-sensitive relapsed *BRCA1*- and *BRCA2*-mutated (germline and/or somatic deleterious

mutations) high-grade serous (HGS) carcinoma of the ovary, fallopian tube, or peritoneum, who are in complete or partial response to platinum-based chemotherapy. Thus, OC patients are now also referred for *BRCA1/2* genetic testing to determine their suitability for PARPi therapy, in the appropriate clinical context. This new indication for *BRCA1/2* genetic testing has also introduced the concept of tumor testing of *BRCA1/2* gene mutations, as this approach can potentially capture both germline and somatic *BRCA1/2* pathogenic variants. As OC is the only indication for which PARPi have been licensed thus far, the remainder of this article will focus on *BRCA1/2* tumor-based testing in OC.

1.2. Ovarian cancer treatment and impact of *BRCA1/2* mutations on treatment outcomes

Platinum and paclitaxel combination chemotherapy has been the first-line standard of care for OC [19], with carboplatin recently replacing cisplatin because it is equally effective and better tolerated. Since 2011, bevacizumab has been approved in Europe in combination with first-line standard of care treatment of patients with advanced epithelial ovarian, fallopian tube, or primary peritoneal cancer. Nonetheless, advanced OC patient prognosis remains poor, with a median PFS and 5-year overall survival (OS) of 14–19 months and 30%–35%, respectively, (ICON 7 and GOG 140), and ultimately most patients will relapse [20–22].

Ovarian cancer comprises different histological subtypes, of which high-grade serous (HGSOC) is the most common, representing ~70% of advanced cases [23–25]. In women unselected for family history, germline *BRCA1/2* mutations have been found in 4%–14% of all OCs, 5%–18% of serous OCs, and ~22% of HGS cases [26–32].

Patients with germline *BRCA1/2*-mutated OC tend to respond well to platinum-based chemotherapy and have a longer median OS than sporadic OC patients [33–39]. The main histological subtype associated with *BRCA1/2* mutations and response to platinum is HGS carcinoma. Other OC histological types have been found to harbor deleterious mutations in other HRR genes [40]; 31% of OCs have a deleterious germline (24%) or somatic (9%) mutation in ≥1 of 13 HRR genes (mutations also associated with primary platinum sensitivity and improved OS).

1.3. Somatic *BRCA1/2* mutations in ovarian cancer

In the Phase II trial of olaparib in patients with platinum-sensitive serous OC, 136 out of 265 patients had a *BRCA1/2* mutation; 20 (15%) were somatically acquired [41]. In another study, *BRCA1/2* mutations were identified in 44 out of 235 (19%) unselected OC tumors, 39% of which were somatic [27]. No additional somatic mutations were detected in tumors from patients with germline mutations, suggesting that loss of heterozygosity (LOH) is responsible in most cases for the bi-allelic inactivation in neoplastic cells. Another study, by The Cancer Genome Atlas (TCGA) consortium, of HGSOC patients found *BRCA1/2* mutations in 64 out of 316 (20%) patients: 9% and 8% were germline *BRCA1* and *BRCA2* mutations, respectively, and 3% were somatic mutations [42].

Whole exome sequencing analysis of the combined germline-somatic landscape in OC in a series of 429 TCGA OCs found germline truncation variants and large deletions across Fanconi pathway genes in 20% of cases [43]. In this analysis, somatic mutations in *BRCA1* and *BRCA2* with potential functional consequences were detected in 4.9% and 2.6% of OCs, respectively.

An analysis of a large set of OC patients for germline (n = 899), somatic (n = 279) and epigenetic alterations (n = 482) in the *BRCA1*, *BRCA2* and *RAD51C* genes revealed deleterious germline *BRCA1* mutations in 32 (3.6%), *BRCA2* in 28 (3.1%), and *RAD51C* in

26 (2.9%) patients [44]. Deleterious somatic mutations in *BRCA1/2* were observed in 3.6% of tumors.

Whether a deleterious mutation has been inherited or acquired somatically, it is thought to be essential that the remaining *BRCA1/2* allele in 17q21 or 13q12, respectively, should be lost in the pre-malignant cells for malignant transformation to occur. In OC tumor samples with deleterious mutations in *BRCA1/2*, LOH of the functional allele was detectable in virtually all cases with *BRCA1* mutations [45–47] and in ~70% of cases with *BRCA2* mutations. These LOH events increase the allelic frequency of the mutation in tumor samples.

1.4. Rationale for *BRCA1/2* tumor testing

When using tumor tissue DNA to detect *BRCA1/2* variants, both germline and somatic *BRCA1/2* mutations are identified. This could be advantageous (time saving and cost saving), as potentially only one test needs to be performed to identify all patients with deleterious *BRCA1/2* variants that may benefit from PARPi treatment. An estimated 10%–20% of OC patients are likely to harbor either a germline or somatic *BRCA1/2* mutation; this approach could facilitate a focused germline testing effort and overall reduction in genetic testing.

2. Considerations and Recommendations for Ovarian Cancer Tumor *BRCA1/2* Genetic Testing

Key recommendations for conducting *BRCA1/2* genetic testing on OC tumor tissue samples are summarized in Table 1.

2.1. Recommendations for *BRCA1/2* tumor testing sample types

Ovarian cancers are characterized by a complex and changing genetic profile [48]; consequently, *BRCA1/2* tumor testing results can potentially vary depending on disease stage and sample site. There is also the possibility that a pathogenic germline or somatic *BRCA1/2* mutation can revert to a functional gene during disease progression, due to development of acquired resistance via somatic revertant mutations [49–54].

In a small study, secondary *BRCA1/2* mutations were identified in 7 out of 10 platinum-resistant OCs and 0 out of 5 platinum-sensitive tumors, leading to the hypothesis that *BRCA1/2* revertant mutations could be associated with treatment resistance [55]. In another study, a second *BRCA1/2* mutation was found in 13 out of 46 (28.3%) recurrent OCs (12 of 13 were platinum resistant), compared with 2 out of 64 (3.1%) primary tumors (one platinum resistant, other unknown platinum response) [50]. A larger study of 92 primary refractory, resistant, sensitive OCs and matched acquired resistant disease used whole-genome sequencing of tumor and germline DNA samples, and found several molecular events associated with acquired resistance, including multiple independent somatic *BRCA1/2* revertant mutations [51].

The limited information available on somatic *BRCA1/2* mutations and response to PARPi from clinical trials relates to analysis of primary ovarian tumors [12,41]. In light of this limited information, it is suggested that *BRCA1/2* tumor testing be performed on primary tumors, as this is the most likely sample type available. However, it should be noted that the analysis of metastatic tissue at the time of progression may provide a more accurate indication of tumors likely to respond to PARPi treatment, due to the evidence supporting the association of revertant mutations and treatment resistance. Surgeons responsible for OC patients need to be made aware of the potential need for tissue testing further down the line and thus the need for adequate collection of tumor

samples prior to surgery (ie, multiple biopsy specimens with a high tumor content).

Formalin-fixed, paraffin-embedded (FFPE) specimens may be used for *BRCA1/2* mutation analysis; this is likely to be the most widely available sample type. Fresh-frozen specimens provide better quality DNA; however, they are not routinely available from most referring centers. Other tissue treatments (eg, alcohol-based, acidic or heavy metal fixatives, or decalcifying solutions) and necrotic samples should be avoided, as they lead to increased DNA degradation. The College of American Pathologists guidelines for Human epidermal growth factor receptor 2 testing in BC can be followed for the fixation of tissue samples [56]. The fixation time should be 8–48 hours, depending on specimen or tissue block size; prolonged cold ischemia time or a fixation duration < 6 hours or > 32 hours increases the likelihood of DNA being of suboptimal quality and unsuitable for *BRCA1/2* testing. Table 2 outlines parameters to consider to ensure a good quality FFPE samples for DNA analysis.

Formalin-fixed, paraffin-embedded sections for DNA extraction should be derived from a single representative block per case and contain ≥30–50 μm depth (eg, 5 x 10 μm or 10 x 5 μm) tissue sections. Appropriately trained pathologists should confirm diagnosis, determine the adequacy of specimens for *BRCA1/2* genetic testing by assessing neoplastic cell content in the specimen, and mark the relevant areas of the tissue to guide macro-dissection as required. Each laboratory should establish the minimum proportion and number of neoplastic cells needed for mutation detection during validation of the methodology. A set of dilutional standards from cells with known *BRCA1/2* pathogenic variants could be used to assess the lower limit of detection.

2.2. Recommendations for DNA extraction from tumor samples

DNA for *BRCA1/2* testing should be extracted from the tumor tissue sample using a validated protocol that ensures sufficient quality and quantity of DNA for the particular methodology, which may widely differ. Tumor samples must have a sufficiently high percentage of neoplastic cells to detect somatic mutations. Generally, a sample for *BRCA1/2* tumor testing is recommended to contain a percentage of neoplastic cells that is at least three times the method's limit of detection (eg, methodology with 5% limit of detection requires the area of tumor sample selected for DNA extraction to contain ≥15% neoplastic cells). This should allow for overestimation of neoplastic cell content, particularly in samples with large areas of inflammation and, to a lesser degree, account for tumor heterogeneity.

The quantity of DNA analyzed can vastly influence result accuracy (eg, analysis of 1 ng of DNA corresponding to the DNA content from ~150 cells can lead to stochastic variation in the sample, increasing the risk of false negative results when the mutation-carrying alleles are not sufficiently represented in the sample or are negatively selected during the process), or false positive results (when artefactual sequences are predominantly amplified during the process). Conversely, 100 ng of DNA corresponds to the DNA content from ~15,000 cells, allowing for a statistically representative sample of tumor DNA. Thus, increasing the quantity and proportion of analyzed tumor DNA improves the likelihood and accuracy of detecting somatic mutations. Although FFPE samples with low tumor content may still allow for the detection of germline mutations (present in all cells) it is always recommended that specimens with sufficient tumor content be analyzed to reliably detect acquired (ie, somatic) mutations.

DNA extraction protocols specific for FFPE tissue are recommended, as they tend to account for the shorter average fragment size compared with high molecular weight (HMW) DNA extraction methods. Fluorometric quantification of DNA should be

Table 1Key recommendations for conducting *BRCA1/2* on ovarian cancer tumor tissue samples.

Recommendation Category	Key recommendations
Sample types appropriate for <i>BRCA1/2</i> tumor testing	Use tissue samples from either primary HGS carcinomas or related metastases; FFPE specimens or fresh-frozen specimens if available Follow The College of American Pathologists guidelines for breast cancer for fixation of tissue samples (Hammond 2010); fixation time 8–48 hours, depending on size of specimen FFPE sections (in total) should be ≥ 50 μ m in depth derived from a single representative block per case, using macrodissection to enrich the neoplastic content when needed Diagnostic H&E stained slides, reviewed by appropriately trained pathologists with molecular pathology experience, should be used for tumor assessment Sample should contain a percentage of tumor cells that is at least three times the limit of detection of the method being used (eg, if a method has a 5% limit of detection, the area of tumor sample selected for DNA extraction should contain at least 15% tumor cells)
DNA extraction from tumor samples	Use a DNA extraction protocol specific for FFPE tissues, deparaffinisation process and recommended starting material should be adhered to Fluorometric quantification of DNA should be undertaken using intercalating dyes (ie, PicoGreen® or Qubit®) as these can more specifically assess the amount of dsDNA in the sample than spectrophotometric methods The quantity of dsDNA ranges from 10–200 ng depending of the procedure used for library preparation
Methodologies for tumor testing	Laboratories should use <i>BRCA1/2</i> test methods that are able to detect mutations in specimens with at least 50% cancer cell content, although laboratories are strongly encouraged to use (or have available an external reference laboratory) more sensitive tests that are able to reliably detect mutations in specimens with $\geq 10\%$ variant allele frequency, corresponding to 10%–20% neoplastic cell content NGS, not Sanger sequencing, should be used for analysis Any method used should have been validated using FFPE tumor samples Amplicons must be shorter when using DNA derived from FFPE material compared with blood samples, and ideally < 150 bp An average sequencing coverage of 500–2000 reads, considering sequencing coverage uniformity, is recommended to avoid false negative assessment and enable detection of somatic mutations when using amplicon-based NGS; however, this is dependent on the assay limits of detection, level of sensitivity desired, and the sample content of neoplastic cells
Avoiding false positive and false negative results	Use duplicate analysis or repeat analysis from the same starting genomic DNA to eliminate artefacts and use sufficient input DNA
Bioinformatics considerations	Any pipeline established and validated on germline <i>BRCA1/2</i> mutations needs to be validated for tumor mutations Collated information is available via a public website that enables the user to search for available information on individual <i>BRCA1/2</i> genetic variants and their current classification of benign or pathogenic at http://brcaexchange.org/ ; ClinVar
Informed consent and ethical considerations	Written information as well as a discussion on the implications for the patient and their families of the test result, which may either be performed personally or via a host of telemedicine technologies, is highly recommended for patients referred for testing Informed consent from patients must be obtained in writing before undertaking germline testing Individual countries vary in their recommendations and legal requirements, and all local criteria must be adhered to
Timing of testing	Tumor testing for <i>BRCA1/2</i> status should be undertaken in order that the result is available when it is clinically relevant to the patient and should factor in the local turnaround time for testing, the potential need for genetic counseling and any associated waiting time, together with other relevant considerations. However, <i>BRCA1/2</i> status can influence all aspects of treatment, thus ideally should be undertaken upon diagnosis

Abbreviations: dsDNA, double-stranded deoxyribonucleic acid; H&E, haematoxylin and eosin; HGS, high-grade serous; FFPE, formalin fixed paraffin embedded; NGS, next-generation sequencing; OC, ovarian cancer

undertaken using intercalating dyes to specifically assess dsDNA quantity, and spectrophotometric quantification should be abandoned. The quantity of dsDNA required ranges from 10–200 ng depending on the library preparation procedure used [57]. It is essential to perform a DNA quality control upfront, to test quality

and amplifiability of the DNA. Laboratories can consider using validated library preparation kits with the *in vitro* diagnostic device CE-marked certification for specific use of testing in FFPE samples, if available. Use of standard DNA samples (commercially available or in-house validated) as positive and negative controls

Table 2

Parameters to consider to ensure good quality FFPE samples for DNA analysis.

Parameter considerations	Recommendations
Fixation method	10% buffered formalin
Fixation time	8–48 hours, depending on size of specimen
Prevention of cross-contamination	Replace knife blades before each new FFPE tissue block is cut Use disposable plasticware to transfer sections to glass slides
Use of DNAzap wipes or bleach on microtome blades	Should be avoided, as are likely to degrade DNA and inhibit PCR
Percentage of neoplastic cells	3 \times the limit of detection of the method
Selection of tumor area	Avoid areas of inflammation or immune infiltrate, areas of necrosis, and selecting many different small regions
Thickness of section	5–10 μ m sections are normally suitable. The amount is dependent on the surface of the dissected area
Macro-dissection	Careful dissection under a magnifier glass is recommended. Pre-wet the scalpel or pipette tip to avoid flakes of tissue coming off the slides
DNA extraction	Any method including a purification step suitable for small DNA fragments can be used
Use of decalcification	Not advised – reduces DNA yield and quality. If decalcification is needed (ie, bone biopsies), EDTA must be used instead of acidic decalcification

Abbreviations: DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; FFPE, formalin fixed paraffin embedded; PCR, polymerase chain reaction

are recommended to assess potential variation in analytical performance over time. Alternatively, clinical samples with known *BRCA1/2* genetic status can be periodically included as controls. Continuous evaluation of the employed procedure in the context of an accreditation program (such as ISO15189 or equivalent), as well as participation in certified external quality assessment (EQA) programs specific for tumor *BRCA1/2* testing from FFPE (ie, EMQN, QulP or UKNEQAS) is highly recommended.

2.3. Advantages and pitfalls of different methodologies for *BRCA1/2* tumor testing

BRCA1/2 are both large genes, and mutations can occur anywhere along the coding region in most populations lacking founder mutations [58]. Thus, it can be more challenging to detect *BRCA1/2* mutations than mutations in oncogenes with known mutation ‘hot spots’ (eg, *KRAS*). Additionally, a significant proportion of *BRCA1* variants consists of large intragenic deletions or rearrangements ($\geq 8\%$ of known *BRCA1* mutations; $> 33\%$ in some northern European countries), which are generally more difficult to detect in DNA derived from FFPE than in HMW germline DNA. The proportion of *BRCA2* large rearrangement is very low ($< 2\%$) [59] and the proportion of these events that are acquired somatically in OC tumors is still unknown. Moreover, the mutations with an impact on splicing should be carefully checked using algorithms (eg, MaxEntScan). Some variants may seem to have no functional impact but in fact effect exon splicing (ie, exon 23 in *BRCA1* [60]) and these events should be carefully analyzed [61,62]. Currently, there is little information in the literature to inform the medical community regarding the decision to test for *BRCA1/2* mutations in OC tumor tissue samples first and then blood, or vice versa. The quality and yield of DNA extracted from FFPE tumors is poorer than DNA extracted from blood and fresh tissue, and tends to be variable [63,64]. FFPE tissue poses specific diagnostic challenges, and fragment sizes of DNA extracted from FFPE tumor tissue is lower, requiring specific adjustment of diagnostic procedures, including expert handling of tissue specimen for DNA extraction, amplicon composition, and diagnostic evaluation. Laboratories should use *BRCA1/2* test methods that are able to detect mutations in specimens with at least $\geq 10\%$ variant allele frequency, corresponding to a minimum content of 10%–20% neoplastic cells.

Next-generation sequencing (NGS) is highly recommended as the sequencing method of choice for *BRCA1/2* tumor testing, due to the quantity of DNA available from tumor samples and the size of

BRCA1/2 coding regions and amplicon composition (160–220 amplicons per test and ideally < 250 bp). Sanger sequencing should be abandoned in tumor testing, as it is not sensitive enough for tumor tissue sample analysis, especially in samples with $< 50\%$ tumor cells, and it also requires a large amount of DNA for the screening of these two large genes. Established methods used for detecting germline *BRCA1/2* mutations from blood samples may not be suitable for tumor tissue testing; it is therefore imperative that any method used must be validated for use on FFPE samples. Amplicon-based NGS approaches for analysis of FFPE material are now commercially available, although each laboratory should internally validate the specific complete pipeline.

The accuracy of tumor *BRCA1/2* testing is influenced by multiple variables, including the specimen's percentage of neoplastic cells and the sensitivity, specificity, and lower limit of detection, as previously discussed. It is important to note that when using amplicon-based sequencing strategies, the number of unique template molecules analyzed is easily overestimated, especially if the amount or quality of input DNA is suboptimal; therefore, extreme care needs to be taken in the analysis of such samples.

The methods used to detect larger deletions or duplications may not directly translate from germline analysis of HMW DNA to FFPE-derived DNA analysis, due to smaller DNA fragment size, DNA chemical modification, chromosomal copy number changes (ie, aneuploidy), and the frequent instability of tumor samples [65]. Furthermore, the frequency of somatically acquired deletions and rearrangements in tumor tissue is unknown; larger cohort studies are required. Bioinformatic approaches using NGS data analysis of copy number variation from capture-enrichment strategies are recommended for identification of large deletions and duplications, if previously validated. Multiplex ligation-dependent probe amplification is a commonly used method for detecting large genomic rearrangements in germline DNA. However, it may not be appropriate for use without adaptation in tumor tissue analysis, as the control reference regions are not suitable to use in the calculations due to aneuploidy present in the tumor cells. Using normalization with intragenic probes allows for detection of intragenic exon deletions and duplication [47]. Such methods need to be extensively validated in the laboratory if all types of variants need to be detected in the tumor sample. Point mutations and small indels are best identified using amplicon and capture-based NGS [66], the advantages and disadvantages of which are outlined in Table 3. An average sequencing coverage of 500 \times –2000 \times reads, considering sequencing coverage uniformity is recommended to avoid false

Table 3

Advantages and disadvantages of amplicon versus capture-based sequencing methods for analysis of tumor tissue samples.

	Advantages	Disadvantages
Amplicon-based NGS	<ul style="list-style-type: none"> Little sample input (DNA from small biopsies) Defined target regions Improved inter-center comparability Higher on target reads Faster sample preparation Simpler workflow and easier bioinformatics Lower complexity in library preparation 	<ul style="list-style-type: none"> Fixation artefacts/PCR duplicates Very difficult to detect large deletions/rearrangements Lower coverage uniformity Mutations at amplicon ends might be problematic Impossible to calculate experimental limit of detection of the assay as unique coverage is generally unknown Vulnerable to false positive results and sensitivity as to required amount of tumor DNA
Capture-based NGS	<ul style="list-style-type: none"> Potential to detect large deletions/ rearrangements (depending on the captured area) Fixation artefacts and/or PCR duplicates are reduced Higher coverage uniformity Possibility to determine amount of unique reads and thus to assess experimental limit of detection Performed better with respect to sequencing complexity and uniformity Less likely than amplicon-based NGS to generate false positive SNVs 	<ul style="list-style-type: none"> More sample input required ($\times 10$) Longer and more laborious methods Increased turnaround times More data output (more detected genomic aberrations and/or interpretation might be more difficult) Complexity of library preparation

Abbreviations: DNA, deoxyribonucleic acid; NGS, next-generation sequencing; PCR, polymerase chain reaction; SNV, single nucleotide variants

negative assessment and enable detection of somatic mutations when using amplicon-based NGS; however, this is dependent on the level of sensitivity desired and the sample content of neoplastic cells. It should also be noted that the number of reads does not necessarily correlate with sensitivity, as many reads may be derived from a single template molecule (ie, polymerase chain reaction [PCR] duplicates). Using a method with single molecule tags overcomes this problem [47]. Moreover, it also depends on the amount of samples loaded onto the sequencer, the type of reagents or chips used for sequencing, and the type of instrument.

Any assay used clinically should be validated in terms of specificity, sensitivity, limit of detection, repeatability (intra-batch variation), and interim precision (inter-batch variation). Laboratory-developed tests need to be fully validated, and commercially available tests also require verification at least (if they are used according to manufacturer's instructions). Assay validation and verification must be performed on typical sample types; in the case of tumor *BRCA1/2* testing, typical samples are OC FFPE, including biopsies and resection specimens. Ideally, a range of typical DNA yields should be evaluated to determine optimal conditions, and to determine any potential assay performance issues if suboptimal conditions are used (eg, low DNA input where artefacts are often more apparent) [67]. In Germany, the Institute of Pathology and Germany's National Accreditation Body (DAKkS) can provide validation, and DAKkS guidelines according to ISO 17020 are available for assay validations. In most other countries, ISO15189 accreditation is necessary.

An EQA testing exercise, involving a number of central European countries, has demonstrated broad, high-quality diagnostic availability of NGS-based *BRCA1/2* mutation testing on FFPE tumor tissue in > 20 centers, providing broad community-based availability of the test [68]. This trial has also shown the diagnostic usefulness of the commonly available NGS platforms and the feasibility of NGS-based testing within time spans required in oncological patient care.

2.4. Avoiding false positive and false negative results

Due to the quality of the DNA extracted from FFPE tissue, artefactual errors caused by the fixation process can result in false positive results. Duplicate analysis or repeat analysis from the same starting DNA can help eliminate artefacts, as they are not generally reproducible due to their stochastic nature [67]. Artefacts can also be reduced if the method being used can discriminate between sense and antisense strands, and/or involve molecular barcodes, which have the additional benefit of being able to assess the number of template molecules analyzed [69–72]. These artefactual errors are a particular issue for amplicon-based sequencing, when DNA concentration is low [67,71], as the rate of PCR duplicates increases with decreased DNA input amounts. This is relevant for FFPE tumor biopsy samples, as the number of PCR duplicates is expected to be higher due to limited specimen material that restricts DNA input amounts and poor sample quality that limits the amount of DNA that can be amplified. However, provided DNA input for the assays is sufficient, many artefacts can be screened out by filtering out variants with an allele frequency < 5%–10%, depending on neoplastic cell content. Mutations should pass quality checks and filter settings, and repetitive variants or artefacts in many samples on the same sequencing run should be excluded.

2.5. Bioinformatics considerations

The bioinformatic process, used to analyze and interpret *BRCA1/2* mutational analysis results, is a critical part of the whole testing process and requires as much consideration, validation, and

accreditation (eg, ISO15189 or CLIA) as the laboratory methods selected to perform the *BRCA1/2* mutation analysis. Individual samples in a pooled reaction must be identified and de-multiplexed according to the indices used in the library preparation and the raw data aligned to the reference sequence. Any variations between the reference sequence and the test samples need to be identified by appropriate variant-calling algorithms, correctly annotated using human genome variant society (HGVS) nomenclature, and classified through interrogation of internal and external databases (list provided in Appendix A). Bioinformatics pipelines are therefore a complex mix of different tools tailored to fit the laboratories in their *BRCA1/2* testing process. A pipeline established and validated on germline *BRCA1/2* testing may not be suitable or may require modification for tumor-tissue-based *BRCA1/2* testing or for testing in a different laboratory with a different methodology, mainly due to the length of amplicons obtained through library design.

Pipelines are typically composed of a combination of commercial software, open-source third-party software and bespoke elements. The amount of validation previously conducted on each of these components is likely to vary; however, commercially available solutions should have undergone some level of verification and so may require less in-house testing. Commercial solutions may be particularly useful for testing laboratories with little or no bioinformatics support. Information security management is important where sensitive information is shared, such as in database queries or variant annotation processes, and local and international legislation relating to this issue must be adhered to. The ISO 27001 is an internationally recognized best practice framework for an information security management system, which is important for bioinformatics platforms that share information among users or manage information from clinical scenarios.

Mutation detection threshold levels that are suitable for detecting germline mutations will need to be modified to detect potentially low-variant frequencies of somatic mutations. There is no set recommendation to where this level should be, as this depends on the performance criteria for the test, including any pre-analytical steps such as DNA yield, test DNA input, and neoplastic cell content.

The bioinformatics processes should be validated on all *BRCA1/2* mutation types. If laboratories do not have access to a sufficient spectrum of data, there is an increasing repository of NGS data in the public domain. This data could be a useful tool to ensure that all variants are being identified, characterized and classified using a laboratory's own pipeline. There are a number of international initiatives for the sharing of genetic data: ENIGMA is a consortium of investigators focused on determining all unclassified variants and variants of unknown significance (VUS) in *BRCA1* and *BRCA2*. The *BRCA* Challenge is a joint initiative of the Global Alliance for Genomic and Global Health and the Human Variome Project, involving several hundred institutes and academic organizations around the world, which aims to pool global available data on *BRCA1/2* genetic variants to further the understanding of *BRCA1/2* genetic variation.

2.6. Reporting tumor *BRCA1/2* results

Recommendations for information to include when reporting *BRCA1/2* testing results are listed in Table 4.

The minimum dataset required in a **clinical report** for tumor *BRCA1/2* testing should include:

- Suitability of the tumor sample, including neoplastic content estimation, cellularity, and whether these are acceptable for the specific testing method

Table 4
Recommendations for information to be included when reporting tumor *BRCA1/2* results.

Information to include	Comments
Histology and grade of the tumor	If available and not previously performed
Percentage of neoplastic content in the analyzed sample and if macrodissection has been performed	Defined as the percentage of neoplastic cells out of the total nucleated cells in the area used for DNA extraction
Procedure used	eg, NGS or Sanger sequencing
Coverage of the analysis	Prevalent mutations vs the recommended whole coding regions including flanking intron sequences
Panel used	Commercial or on demand or multipanel gene (eg, panels including more than <i>BRCA1/BRCA2</i> genes)
Minimum coverage and guaranteed analytical sensitivity with the employed methodology	Any regions for which there was insufficient sequencing read coverage (eg, 33 reads, dependent on assay limits of detection) should be declared to avoid false negative assessment
If the analysis includes all types of alterations or just single point mutations or small indel alterations	eg, 'large rearrangements/deletions cannot be detected using this methodology'
Sequencer	Models include: MiSeq, NextSeq 500, PGM, IonProton
Bioinformatic tools employed	Freeware or commercial; indicate if they have been validated or not for this type of analysis. If the tools are web based, include the https address
Requested databases for grading of detected mutation	UMD and ClinVar-DB, etc.
Variant allele frequency	It is important to correlate with neoplastic tumor content and potential LOH, which is present in the vast majority of cases
Explanation of therapeutic consequence of mutations according to the classification of the variants	Mutation class 4 or 5 –pathogen = possibility of therapy with PARPi; mutation class 1–3 or wild type = no therapeutic consequence and should not be reported (see text for VUS)
A recommendation of germline analysis and genetic counseling in cases of pathogenic mutations	Germline analysis should be always considered if not already performed
Accreditation of the laboratory	ISO15189, CAP, CLIA or equivalent
Participation in EQA	For example: EMQN, QuIP, UK NEQAS

Abbreviations: CAP, College of American Pathologists; CLIA, Clinical Laboratory Improvement Amendments; DNA, deoxyribonucleic acid; EMQN, the European Molecular Genetics Quality Network; EQA, external quality assessment schemes; LOH, loss of heterozygosity; NGS, next generation sequencing; PARPi, poly (adenosine diphosphate-ribose) polymerase inhibitor; PGM, personal genome machine; QuIP, Arbeitsgruppe für Qualitätssicherung in der Molekularpathologie der Österreichischen Gesellschaft für Pathologie; UK NEQAS, United Kingdom National External Quality Assessment Service; UMD, Universal Mutation Database; VUS, variants of unknown significance

- Targets analyzed (ie, *BRCA1* and/or *BRCA2*)
- The regions covered for each gene (eg, coding region only or intronic and exonic regions)
- Overall results: either pathogenic or deleterious variants present or absent
- Mutation details (when present): cDNA and amino acid change according to HGVS nomenclature
- Reference sequence, including version used for annotation and HGVS nomenclature
- Summary and interpretation: including pathogenic or likely pathogenic classification of the identified variants. Non-pathogenic variants should not be reported, although the laboratory may keep a record of these. Variants of unknown significance should be reported separately and clearly indicate the lack of sufficient clinical or biological evidence. Information regarding the potential therapeutic implications should be included when possible.

2.7. Should genetic testing be limited to *BRCA1/2* testing?

Currently, genetic testing in OC should be limited to *BRCA1/2* for clinical- and therapeutic-driven decisions, as indications to date are restricted to these genes. However, additional genes involved in the HRR pathway are being evaluated in a number of clinical trials (NCT02422498, NCT02489058, and NCT01434316) and are likely to become part of the standard testing methodology in the future [73]. A recent study has shown increased efficacy of PARPi in OC with HRD without a germline *BRCA1/2* mutation [14].

3. Integrating *BRCA1* and *BRCA2* Tumor Testing In The Patient's Pathway

Commissioning strategies in different countries relating to both *BRCA1/2* tumor and germline testing are diverse. In October 2014, the Society of Gynecological Cancer recommended that all women diagnosed with ovarian, fallopian tube, or peritoneal carcinoma,

regardless of age or family history, should receive genetic counseling and be offered *BRCA1/2* genetic testing [74]. Many individual country guidelines now also recommend offering *BRCA1/2* genetic testing to OC patients irrespective of a family history of BC or OC, an approach that may in fact be cost-effective for universal healthcare systems [75] (Appendix B) [11,76–84]. Although the prevalence of *BRCA1/2* pathogenic variants is highly associated with a familial history, with a > 75% probability of a mutation in women with a strong BC or OC familial history [85], the availability of PARPi and lack of accurate family history for many patients make it appropriate to refer all OC patients for genetic testing.

3.1. Germline v tumor *BRCA1/2* testing from the clinical perspective

It is necessary to determine the *BRCA1/2* status of an OC patient in order to be able to determine eligibility for PARPi therapy, and testing the tumor samples can in principle detect both somatic and germline variants. The limitations of tumor testing must, however, be considered. It is difficult to distinguish between germline and somatic mutations by analyzing tumor tissue in isolation and it is therefore inappropriate to draw any conclusions concerning familial risk based on this analysis alone. Any patient found to have a deleterious *BRCA1/2* mutation in a tumor specimen should be offered genetic counseling to undergo germline analysis to assess the existence of the mutation in germline DNA, a finding affecting familial risk. On the other hand, genetic analysis in blood samples can only identify germline mutations, missing approximately 3%–5% of patients who have an acquired *BRCA1/2* pathogenic variant [12,41] and who could also benefit from PARPi therapy. The advantages and disadvantages of *BRCA1/2* tumor testing versus blood germline testing are outlined in Table 5.

3.2. When in the patient journey should *BRCA1/2* testing be carried out?

Tumor testing for *BRCA1/2* status should be undertaken in order for the result to be available at a clinically relevant time frame,

Table 5
Advantages and disadvantages of *BRCA1/2* tumor testing versus blood germline testing.

	Tumor <i>BRCA</i> testing	Blood <i>BRCA</i> testing
Advantages	Can detect both somatic and germline mutations Identifies a greater number of patients who may benefit from PARPi therapy Potentially requires less extensive genetic counseling at the outset, and less involvement for the wider family Reverted <i>BRCA1/2</i> mutations can identify patients resistant to treatment	Validated methods are available and professionals are experienced in testing and interpreting variants Patient protocols, pathways and procedures are well established Evidence is strong for the association between <i>BRCA</i> germline mutations and response to PARPi therapy Sample is easily obtained and contains high-quality DNA Analysis feasible in 100% of cases
Disadvantages	Validated methods not yet widely available Types of mutations not well defined Only preliminary data are available on the response to PARPi associated with somatic mutations Sample with sufficiently high percentage of tumor cells/quality of DNA may be hard to obtain, leading to the need for repeat testing and/or biopsy Analysis not always possible for technical reasons. Investment in new NGS technology may be required as most traditional methods are unsuitable due to limited DNA Requires additional expertise in pathology to determine sample adequacy	Does not identify patients with somatic mutations who could benefit from PARPi therapy Genetic profile of the tumor may change with disease progression and chemotherapy

Abbreviations: NGS, next-generation sequencing; PARPi, poly(adenosine diphosphate-ribose) polymerase inhibitor

factoring in the local testing turnaround times, the potential need for genetic counseling, and other relevant considerations (Figure 1). Referral timing for OC patients' genetic testing is not specified in many guidelines, but where it is, it is either recommended at diagnosis (Italy [76,82]) or as soon as possible during the first treatment cycle (Austria [83]; Belgium [77]). Given the experience thus far in germline *BRCA1/2* testing in OC and microsatellite instability testing of colorectal cancer patients for Lynch syndrome, most patients are willing to undergo testing early on in their management pathway.

Obtaining a tumor tissue sample for *BRCA1/2* genetic testing earlier on in the patient pathway may be advantageous. When testing is for treatment decisions, it is desirable to have the test within a maximum of 30–40 day of requesting. For countries considering *BRCA1/2* testing to be initially tumor-based rather than germline-based, a proportion of OC patients may never undergo *BRCA1/2* testing if testing is limited to the time when PARPi may be used (eg, patients that do not fulfil the approved indication or have unsuitable tissue specimens or are deceased prior to tumor testing). And for those OC patients in this latter group who may harbor a germline mutation, a lack of *BRCA1/2* genotyping may have grave implications for family members.

All of the above considerations should of course be reviewed if and when PARPi treatment could be considered for first-line treatment.

3.3. Informed consent and ethical considerations

Tumor testing for somatic mutations to better define therapeutic options is a well-accepted concept and practice in the management of other malignancies (eg, *EGFR* and *ALK* testing in lung cancer or *KRAS* and *NRAS* in colorectal cancer). However, these mutations and genetic variants are clearly somatic by nature and do not have any ethical ramifications. *BRCA1/2* tumor tissue genotyping poses different challenges given that an identified tumor *BRCA1/2* mutation may represent a germline *BRCA1/2* mutation in the majority of cases. Therefore, it is important that patients are adequately counseled prior to testing regarding both the personal clinical implications of the test results and the possible impact of *BRCA1/2* genotyping on asymptomatic family members. Informed patient consent and/or institutional review board approval is, at least to some degree, required prior to performing any genetic analysis.

The legal framework in different countries for the performance of genetic testing is heterogeneous. Germline genetic testing is protected or restricted by law and pre-test genetic counseling is mandatory in many countries. Counseling for germline *BRCA1/2* testing is traditionally performed by genetic counselors or medical geneticists who specialize in oncogenetics. Due to increased demand on services, some European centers have adopted an oncogenetic pathway for germline *BRCA1/2* testing, in which

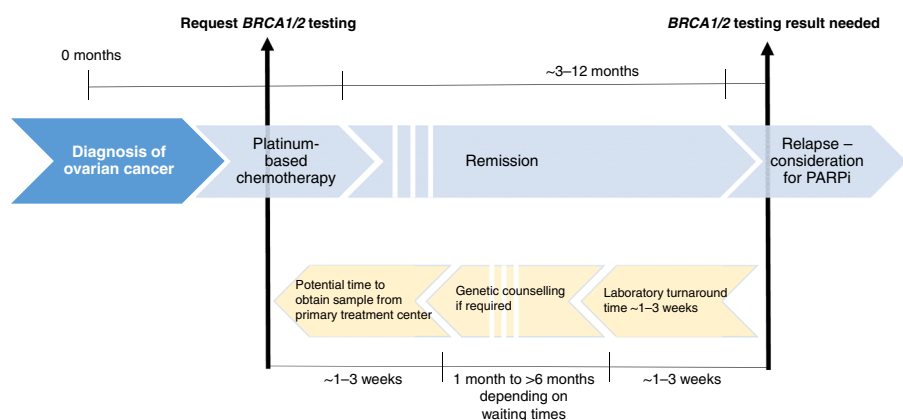


Figure 1. Timeline of factors to consider when determining the time to request *BRCA* tumor testing.

counseling and consent for genetic testing takes place in the oncology unit, with support from the genetic department, provided by gynecologists or oncologists with training in Germany and Austria [83] and oncologists with specialist training in the UK [86]. Advanced Nurse Practitioners and Clinical Nurse Specialists are another integral part of the oncology team, who, with the support of the genetics and/or oncology team, are now also involved in patient consenting for testing of somatic or germline *BRCA1/2* mutations [87]. For tumor *BRCA1/2* testing, counseling and obtaining consent in the context of oncology clinics is likely to be a more practical option than referring all patients for pre-surgical genetic counseling at the relevant genetics institute. Because of this ever increasing need, it is likely that new models of counseling will be adopted in the near future [88]. Appropriate pre-test counseling is required prior to testing even if analysis is only initially being performed on a tumor tissue sample, because germline testing should be offered to the patient and family members in the event that a mutation is detected. When a hereditary OC is identified, the patient's relatives have possibilities for prevention and should be informed about undergoing genetic testing and treatment options, particularly prophylactic surgery. This type of genetic counseling is also required to be non-directive in several countries (eg, Austria, Germany, Netherlands).

4. Conclusion

With the recent approval of olaparib for platinum-sensitive OC with *BRCA1/2* deleterious mutations, a review in *BRCA1/2* testing strategy is warranted in most countries. The current paper reviewed the different national and local strategies for *BRCA1/2* testing, in particular how germline testing may now be offered to all women with OC based on a > 10% prevalence of *BRCA1/2* mutations in this unselected population, raising to > 15% in patients with HGSOC. Based on the European license for olaparib, genetic testing of tumor samples is needed to capture all possible patients that may benefit from PARPi treatment. This new testing paradigm poses some challenges, in particular the technical and analytical difficulties of analyzing chemically challenged DNA derived from FFPE specimens.

The question of whether germline or tumor testing needs to be performed in sequence or in combination will likely be answered differently in different countries, as such strategy is impacted by local or national regulatory elements as well as existing clinical pathways. In any case, identification of deleterious *BRCA1/2* mutations in tumor tissue requires subsequent germline testing to assess the inheritability of such variation after appropriate genetic counseling. Moreover, cascade testing of relatives of patients with a germline mutation should be available. Similarly, OC patients without deleterious germline *BRCA1/2* mutations will require tumor testing to identify the additional 3%–9% of patients that could benefit from PARPi. Tumor *BRCA1/2* testing is currently available in most European countries by different methodologies, although all of them make use of the current NGS technology available. It is important to note that stringent validation of both the NGS methodology and the bioinformatics pipelines for analysis of NGS data derived from FFPE-extracted DNA is required, as this poses different challenges to germline testing, including the influence of neoplastic cell content in the variant allele frequency and the potential artefacts derived from chemical modification of the DNA during the processing of the specimen.

Regardless of the testing algorithm and methodology chosen, it is paramount that any change in practice is linked to the patient's pathway to ensure that all patients have access to the tests required for their care. This means both germline and tumor testing need to be taken into consideration when designing a

new testing strategy, as there is a risk of patients missing important information if one of the tests is not performed as and when appropriate. Such pathways would likely be affected by the requirements for informed consent prior to germline and/or tumor testing, and national and international policies and guidelines will be required to establish best practice for OC patients in the context of PARPi therapies. Furthermore, new scientific evidence regarding the potential benefit of PARPi for OC patients without *BRCA1/2* mutations, but with deleterious variants in other HRR genes, is becoming available, and clinical trials assessing the value of other biomarkers are being performed. It is therefore likely that a completely new and comprehensive testing strategy will be required in the next few years for the clinical management of OC patients, in line with the advances of precision medicine in other oncology areas.

Conflicts of interest

DGC has received honoraria and research funds from AstraZeneca.

MJLL has received honoraria and research funds from AstraZeneca and is a member of the onconetwork consortium of Life Technologies.

GE was, at the time of the study, an employee of AstraZeneca, plc.

CS, RS, RB have received honoraria from AstraZeneca.

EF has no conflict of interest to declare.

IRC has received honoraria for lecturing and advisory board attendance from Pharmamar, Roche and AstraZeneca.

JAL has no conflict of interest to declare.

SB has received honoraria for advisory board attendance from AstraZeneca and Clovis Oncology.

CJvA has received research funds from AstraZeneca.

VE has acted as consultant scientist for Thermo Scientific/IonTorrent.

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Appendix A. Supporting information

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